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Evaluation of intracellular and extracellular domoic acid content in *Pseudo-nitzschia multiseries* cell cultures under different light regimes

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ABSTRACT

Pseudo-nitzschia multiseries is a diatom species associated with the production of domoic acid (DA), a water soluble neurotoxin that is easily transferred up in the food web, causing devastating effects on top marine organisms and humans. Despite studies on *Pseudo-nitzschia* are relevant to human health safety, partitioning of marine toxins between intracellular and extracellular fractions are poorly documented. This study aimed to determine the growth rates and DA content, both intracellular and extracellular, of *Pseudo-nitzschia multiseries* cultures at three different light settings (15, 120 and 560 µmol m^{-2} s^{-1}). The optimal conditions for cell growth were observed at 120 and 560 µmol m^{-2} s^{-1}, whereas DA production was observed in *P. multiseries* at 15 and 120 µmol m^{-2} s^{-1}, ranging between 0.18-2.56 and 0.16–3.5 pg DA $cell^{-1}$, respectively. Higher intracellular DA concentrations were found during the senescence phase at low light intensity and during the exponential phase at medium light intensity, while higher concentrations of dissolved DA were found at low and medium light intensities in the senescence phase reaching 3 and 10 ng DA mL^{-1} respectively. The amount of toxin released into the culture medium represents the most important fraction ranging between 63 and 98% during the exponential phase and nearly 99% during the senescence phase. In contrast, under low light intensity, dissolved DA was detected in the culture medium only during the senescence phase. This study confirms the importance of light intensity on DA production and clearly shows that dissolved domoic acid is an important fraction in *Pseudo-nitzschia* cultures, suggesting with the careful assumptions of results from static cultures extrapolated to bloom situations that waterborne exposure of marine organism should be considered during blooms of *Pseudo-nitzschia multiseries*.

1. Introduction

Pseudo-nitzschia genus is a marine planktonic chain-forming with a worldwide distribution, in particular on eastern boundary upwelling systems. The blooms are seasonal, occurring during spring and fall, when irradiance and temperature conditions are relatively low (Bates et al., 1998; Mercado et al., 2005; Trainer et al., 2012). As commonly observed in diatoms, *Pseudo-nitzschia* spp. grow rapidly in high-Si regimes and their blooms are associated with upwelling events. The increasing availability of nutrients combined with the mixture of water column allow diatom cells to remain closer to the surface, where they have greater light availability, thus stimulating blooms occurrence (Anderson et al., 2006; Lund-Hansen and Vang, 2004; Trainer et al., 2002).

Studies on *Pseudo-nitzschia* blooms have increased in the last years since this diatom genus was reported to produce domoic acid (DA) (Stonik et al., 2011; Thessen and Stoecker, 2008; Trainer et al., 2012). Among the 19 species known to produce DA, mostly from the seriata complex (Teng et al., 2016), only *Pseudo-nitzschia multiseries*, *P. pseudodelicatissima*, *P. australis* and *P. seriata* have been associated with human poisoning incidents (Amnesic Shellfish Poisoning) or closures to shellfish harvesting (Fehling et al., 2004). To ensure the safe consumption of shellfish, many research and monitoring programs for the detection of *Pseudo-nitzschia* in seawater and DA in shellfish have been implemented. In addition to contamination of shellfish resources, DA can have a profound impact in the marine environment due to its bioaccumulation in the food web, which in extreme cases leads to massive deaths of top predators, such as marine mammals and seabirds

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(Sierra Beltrán et al., 1997; Lefebvre et al., 2002).

Among the several environmental parameters affecting DA production, light is essential to ensure toxin production, since it provides the necessary energy for its biosynthesis and controls photophosphorylation activity, nitrogen reduction and carbon assimilation (Pan et al., 1998). DA synthesis is considered to occur when the photosynthetic rate, cellular materials and growth rate are decreasing, and cells are metabolically inactive or physiologically stressed. In this context, the unbalanced variation in cellular materials and low photosynthetic activity may be linked with DA production (Pan et al., 1991). The role of light in DA production is twofold: on one hand, DA is produced as a mean to control the photosynthetic energy flow within the cell (Pan et al., 1998), while on the other hand, DA absorbing in UV-A portion of the UV-spectrum enhance carbon fixation (Gorga et al., 2002). Wells et al. (2005) believe that all species of *Pseudo-nitzschia* will prove to be toxigenic, given the proper growth conditions and sensitive-enough detection protocols. In batch cultures, DA has been often found during the stationary phase when DA is accumulated as consequence of stopping cell division (Cusack et al., 2002; Kotaki et al., 2000). Other authors found that DA may be produced during the late exponential phase, being a period of transition when some cells have stopped growing while others are still dividing (Bates, 1998; Pan et al., 2001).

In addition to the toxin levels found within in the diatom cells, *i.e.* intracellular DA, the toxin can be released into the culture medium as dissolved DA via excretion processes or by cell lysis at the end of the stationary phase or senescence (Mos, 2001), which may represent a different route of exposure for marine organisms. Most studies have been focusing on toxins incorporation through feeding of toxic algal cells. However, exposure to toxins in their dissolved form may particularly affect early life stages of many organisms (Vasconcelos et al., 2010). A significant reduction of scallops (*Pecten maximus*) larvae growth and survival after exposure to dissolved DA has been reported, suggesting that DA exposure might possibly influence *P. maximus* recruitment (Liu et al., 2007).

Therefore, it is important to understand how light intensity affects the growth behavior and consequently DA production of *Pseudo-nitzschia* species. In this context, the aim of the present study is to determine the growth rates and DA production, both intracellular and dissolved in the culture medium of *P. multiseries* under different light regimes.

2. Material and methods

2.1. Strain isolation and species identification

The experiment was carried out using a clonal non-axenic strain of *P. multiseries*, isolated from a bloom that occurred in Cascais, Portugal, in October 2014. The strain was identified by PCR-based assay following Penna et al. (2007) methodologies.

2.2. Culture conditions

Batch cultures of *Pseudo-nitzschia multiseries* were grown and maintained in sterile-filtered seawater with f/2-medium enriched with silica (Andersen, 2005) in 2L borosilicate Erlenmeyer flasks with triplicates by light treatment. The growth conditions were as following: 19 °C, 33–34 PSU, with aeration and illuminated with cool-white fluorescent lights on a light:dark cycle of 12:12 h. The light settings were defined based on the range of light daily measured between April and June that corresponds to the most favorable period for *Pseudo-nitzschia* blooms in the Portuguese coast. Irradiance values were calculated based on the light attenuation coefficient and taking into consideration that blooms are sub-superficial (below 3 m depth) (Silva et al., 2009). The light settings used were 15, 120 and 560 µmol photons m^{-2} $s^{-1,}$ considered low, medium and high light intensity, respectively. The 120 µmol photons m^{-2} s^{-1} represents the usual light intensity in the Portuguese coast during *Pseudo-nitzschia* spring blooms. The 15 and 560 µmol photons m^{-2} s^{-1} were used to represent the lowest and highest light intensities during this season.

2.3. Growth rate determination

The growth rate determination started with an initial concentration of 80,000 cells L^{-1}, representing the initial phase of the bloom formation. For cell counts, the culture was gently mixed before 3 mL subsamples were taken from each flask, daily. Cells were preserved with Lugol's iodine solution and counted in triplicate, using an inverted microscope (Leica DMi8) at 100x magnification. Cells were preserved with Lugol's iodine solution and counted in triplicate, using an inverted microscope (Leica DMi8) at 100x magnification. Cells were quantified with a Sedgewick–Rafter counting chamber for low concentrations and with a Palmer-Maloney counting chamber for higher concentrations and only living cells were counted (Lund et al., 1958). Growth rates (in days) were calculated according to the formula (Lelong et al., 2011):

$$\mu = \ln(N_1/N_0)/\Delta t$$

With µ = growth rate (d^{-1}), N_0 = initial cells concentration, N_1 = final cells concentration and Δt = time interval between counts.

2.4. Domoic acid extraction

Intracellular and dissolved DA were analyzed daily in 100 mL of culture in the beginning of the exponential phase and in 20 mL during the late exponential and stationary phases, by filtering through Whatman GF/C glass fiber filters (47 mm diameter) to separate the cells (intracellular DA) from the culture medium (dissolved DA). All samples were stored at −20 °C until extraction.

For intracellular DA extraction, 3 mL methanol (methanol: water, 50:50, v/v) was added and cells were disrupted by sonication (Vibra-Cell - Sonics & Materials, Inc) for 3 min to release intracellular DA into solution. Samples were homogenized by vortex, centrifuged during 10 min at 4000 rpm and then filtered with a 0.2 µm GHP syringe filter into an LC vial for LC–MS/MS analysis.

For extracellular (dissolved) DA extraction, a 2 mL aliquot of seawater sample was acidified with 0.7 mL of 2% formic acid/5% methanol/water (2:5:93, v/v/v) to yield 0.5% formic acid in the sample. After vortexing, samples were desalted and extracted for DA by solid phase extraction (SPE) using Oasis HLB cartridges. The SPE column was conditioned with 5 mL methanol followed by 5 mL ultrapure water using a vacuum manifold. Then, 2 mL of the acidified seawater sample was loaded to the SPE column, followed by 5 mL of ultrapure water to rinse the sample tube and the SPE column. The DA was eluted dropwise with 2 mL of methanol into a glass vial and analyzed by LC-MS/MS (Wang et al., 2007).

2.5. Determination of domoic acid via liquid chromatography

The LC-MS/MS equipment consisted of a Dionex Ultimate 3000 LC system (Thermo Scientific) coupled to a TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo Scientific). The chromatographic separation was conducted using an Accucore RP-MS column (150 × 2.1 mm, 2.6 µm), protected with a guard column Accucore RP-MS (10 × 2.1 mm, 2.6 µm). Elution was achieved using a binary eluent system: eluent A was water with 2 mM ammonium formate and 50 mM formic acid, and eluent B was 95% acetonitrile with 2 mM ammonium formate and 50 mM formic acid. A binary elution gradient was used at a flow rate of 0.3 mL min^{-1} as follows: 0–2 min, gradient from 70 to 5% eluent A; 2–4 min 5% eluent A; 4–6 min gradient from 5 to 70% eluent A; 6–8 min, 70% eluent A. Three MRM transitions from the protonated DA ion were monitored: *m/z* 312 > 266, *m/z* 312 > 248, and *m/z* 312 > 161.

Domoic acid certified reference standard (CRM-DA-g) purchased